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A barcode-based outlook on the Western European *Setina* Schrank, 1802 (Lepidoptera, Erebidae, Arctiinae)

Antonio S. Ortiz[1], Rosa Maria Rubio[1], Juan Jose Guerrero[1], Manuel Garre[1], José Luis Yela[2]

1 *Departamento de Zoología y Antropología Física, Universidad de Murcia, Murcia, Spain*

2 *Grupo DITEG, Facultad de Ciencias Ambientales, Universidad de Castilla-La Mancha, Toledo, Spain*

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Corresponding author: Antonio S. Ortiz (aortiz@um.es)

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Abstract

The European representatives of the erebid genus *Setina* Schrank, 1802 exhibit considerable intraspecific and interpopulation variability. In this study, their taxonomic status is reassesed using an integrative approach that combines a review of the existing literature, morphological examination, and analysis of mitochondrial DNA sequences from the protein-coding cytochrome *c* oxidase subunit I gene (COI5P). The major European lineages of *Setina* appear to form three species complexes: *Setina irrorella* (Linnaeus, 1758), including the subspecies *flavicans* (Geyer, 1836), distributed from Spain to European Russia; *Setina roscida* (Denis & Schiffermüller, 1775), including the subspecies *cantabrica* de Freina & Witt, 1985 and *kuhlweini* (Hübner, 1824), ranging from the Iberian Peninsula to central Siberia; and *Setina aurita* (Esper, 1787) which is currently regarded as restricted to the Alps.

Key Words

Arctiinae, cryptic diversity, DNA barcoding, Erebidae, Europe, evolutionary history, Iberian Peninsula, Lepidoptera, morphology, phylogeography

Introduction

The genus *Setina* Schrank, 1802 (Erebidae, Arctiinae) is taxonomically complex due to pronounced intraspecific and interpopulation variability, which may indicate relatively recent or ongoing speciation processes. As a consequence, the male genitalia are remarkably uniform throughout the genus and exhibit few species-specific characters. The most informative diagnostic features are found in the structures of the valvae and the architecture of the vesica. In females, the main distinguishing characters involve the shape and size of the ductus bursae and the appendix bursae. Species of *Setina* are small to medium-sized and display clear sexual dimorphism in the shape and size of wings, the length and width of the abdomen, and the configuration of the antennae. The forewings are typically yellowish with dark dots or lines, whereas the hindwings are usually paler and bear a marginal row of dark spots, which may be partly reduced or absent. Members of the genus inhabit sandy and rocky slopes and subxerothermic grasslands, and may occur at elevations exceeding 3000 m in the Alps. Adults are on the wing from late spring to early autumn (de Freina and Witt 1987; Leraut 2018).

The genus *Setina* is currently considered to comprise six species in Europe. Reflecting the high variability within the genus, numerous synonyms and fourteen subspecies were listed by Witt and Ronkay (2011): three subspecies of *Setina irrorella* (Linnaeus, 1758); two of *Setina flavicans* (Geyer, 1836); three of *Setina alpestris* Zeller, 1865; four of *Setina aurita* (Esper, 1787), in its current sense; two of *Setina roscida* (Denis & Schiffermüller, 1775); and the Iberian endemic *Setina cantabrica* de Freina & Witt, 1985. Earlier, Trawöger (1991), in a study on hybridization within the genus, recognized up to eight subspecies of the so-called *S. aurita*, although he distinguished only three sympatric species: *S. irrorella*, *S. aurita* and *S. roscida*. Subsequently, Parenzan and

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Scalercio (2001) proposed a taxonomic grouping based on male genital morphology and ecological traits. They recognized the *S. irrorella* group—comprising *S. irrorella*, *S. alpestris* and *S. aurita*—characterized by a spiny clasper, and the *S. roscida* group—comprising *S. roscida*, *S. cantabrica* and *S. flavicans*—distinguished by a concave clasper. Later, Leraut (2006) treated *S. flavicans* as a subspecies of *S. irrorella*, *S. alpestris* as a subspecies of *S. ramosa* (Fabricius, 1793), and *S. cantabrica* as a subspecies of *S. roscida*. More recently, Leraut (2018) noted that populations of most *Setina* species in Western-central Europe remain poorly known and that the nomenclature of several taxa is still unsettled. He reviewed various taxonomic arrangements proposed by earlier authors, including Rougeot and Viette (1978), Burmann and Tarmann (1985), de Freina and Witt (1987), Leraut (2006) and Witt and Ronkay (2011). In addition, Leraut (2018), following observations already noted by Burmann (1955), suggested that the considerable morphological variability observed in the genus may partly reflect phenotypic plasticity related to elevation, as some species—particularly *S. irrorella*—occur from sea level to more than 3,600 m. In that same work, Leraut (2018) proposed the use of the name *S. ramosa* in place of the commonly used *S. aurita*, arguing that the latter is a junior homonym of *Noctua aurita*, now *Trigonephra aurita* (Fabricius, 1787) (Noctuidae: Oncocnemidinae). However, this interpretation overlooked that *Setina ramosa* (Fabricius, 1793) was originally described in the genus *Bombyx* and therefore constitutes a more recent primary homonym of *Bombyx ramosa* Esper, 1786, currently recognized as *Calliergis ramosa* (Esper, 1786). Finally, Leraut (2018) considered *S. irrorella*, *S. roscida*, *S. ramosa* and *S. alpestris* as valid species.

The present investigation was prompted by results obtained during an effort to DNA-barcode all macroheteroceran Lepidoptera species occurring in the Iberian Peninsula., This work revealed that Iberian individuals of the genus *Setina* share barcode haplotypes with populations from Central Europe. In this study, we analyze mitochondrial COI sequences together with the geographical distribution of the identified lineages to evaluate differences among European population groups and to provide new insights into their taxonomic relationships. Our research aims to examine all taxa within a broader European context, avoiding interpretations centered on particular regions—such as Central Europe on the one hand and south-western Europe on the other—which in the past have sometimes led to the over- or underestimating of the taxonomic status of certain taxa.

Material and methods

Morphological study

The present study was based on the morphological analysis of 23 individuals (18 *flavicans*/5 *cantabrica*) with Sample-ID code IBLAO in Table 1 collected from different localities in Spain while the rest of the taxa collected in areas other than the Iberian Peninsula have not been morphologically reviewed (Fig. 1). These adult specimens were examined externally to evaluate possible differences in their colouration, spot size and wing shape based on the taxonomic key provided by Witt and Ronkay (2011) and Ylla et al. (2010) and were dissected using a standard procedure (Fibiger 1997) with minor modifications. Genital structures were examined using a Zeiss Stemi 508 stereomicroscope with a Zeiss Axiocam 208 digital camera and were compared with those published by Witt and Ronkay (2011) and Ylla et al. (2010). Specimens were deposited in the Research Collection of Biología Animal from the Departamento de Zoología y Antropología Física (University of Murcia, Spain) and the research collection of José Luis Yela (University of Castilla-La Mancha, Toledo, Spain).

Molecular study

Twenty-three adult specimens of *Setina* were processed and sequenced at the Canadian Centre for DNA Barcoding (CCDB, Guelph) to obtain DNA barcodes using the standard high-throughput protocol described by de Waard et al. (2008) available at www.dnabarcoding.ca/pa/ge/research/protocols. The dataset was complemented with sixty-three publicly available sequences downloaded from The Barcode of Life (BOLD: www.boldsystem.org) (Ratnasingham and Hebert 2007). In total, the analysis included 86 *Setina* COI sequences from Europe that were freely available in BOLD and exceeded 500 bp in length, allowing their use for the analysis of sequence divergence and phylogenetic tree constructions (Table 1, Fig. 1). Original species identifications assigned by the entomologists responsible for the sequences were retained for subsequent discussion. Voucher data, GPS coordinates, images, sequences, and trace files are publicly accessible through the dataset (dx.doi.org/10.5883/DS-SETINA) on BOLD.

Sequence divergences for the barcode region (mean pairwise distances in %) were calculated using the Kimura 2-parameter (K2P) model (Kimura 1980) and interspecific genetic variation was estimated using the analytical tools implemented in BOLD. All newly generated and publicly available sequences were downloaded and aligned using the K2P algorithm in MEGA6 (Tamura et al. 2013). Bootstrap support values were calculated with 1000 replicates. An initial distance-based Neighbor-joining (NJ) tree was constructed in MEGA6, while Maximum Likelihood (ML) analysis was performed using FastTree 2.1.11 (Price et al. 2009, 2010). To root the phylogenetic tree, *Stigmatophora micans* (Bremer & Grey, 1852)—a species belonging to the only genus systematically related to the subtribe Endrosina (tribe Lithosiini)—was selected as the outgroup (Scott and Branham 2012). For the estimation of COI divergences, all sites were included using the pairwise deletion option. The number of haplotypes was calculated using DnaSP 5.10 (Rozas et al. 2003).

Table 1. Taxon names (original uploader identification), BIN (Barcode Index Number), BOLD accession numbers for the specimens used in distance estimations (Process and Sample ID), haplotype and locality information (Country and Region).

Taxon	BIN	Process ID	Haplotype	Country	Region
Setina roscida	BOLD:ABZ1427	LEASS782-17	Hap_01	Austria	Salzburg
Setina roscida	BOLD:ABZ1427	LEFIL576-10	Hap_01	Sweden	Ol
Setina roscida	BOLD:ABZ1427	LEAST1150-17	Hap_02	Austria	Osttirol
Setina roscida	BOLD:ABZ1427	LEAST1151-17	Hap_03	Austria	Osttirol
Setina cantabrica	BOLD:ABZ1427	IBLAO1949-21	Hap_04	Spain	Cantabria
Setina cantabrica	BOLD:ABZ1427	IBLAO1959-21	Hap_04	Spain	Cantabria
Setina cantabrica	BOLD:ABZ1427	IBLAO1316-20	Hap_05	Spain	Asturias
Setina cantabrica	BOLD:ABZ1427	IBLAO1317-20	Hap_05	Spain	Asturias
Setina cantabrica	BOLD:ABZ1427	IBLAO822-12	Hap_05	Spain	Asturias
Setina kuhlweini	BOLD:ABZ1427	POESE458-22	Hap_06	Germany	Branderburg
Setina roscida	BOLD:ABZ1427	ABOLA548-14	Hap_06	Austria	Niederoesterreich
Setina roscida	BOLD:ABZ1427	LON7240-18	Hap_06	Croatia	Zadar Country
Setina roscida	BOLD:ABZ1427	LON7241-18	Hap_06	Croatia	Zadar Country
Setina roscida	BOLD:ABZ1427	GBLAB1853-14	Hap_07	Germany	Bavaria
Setina roscida	BOLD:ABZ1427	ABOLA549-14	Hap_08	Austria	Niederoesterreich
Setina roscida	BOLD:ABZ1427	LEATG031-14	Hap_09	Italy	Suedtirol
Setina roscida	BOLD:ABZ1427	LEATG032-14	Hap_09	Italy	Suedtirol
Setina flavicans	BOLD:AAC0260	IBLAO1968-21	Hap_10	Spain	León
Setina flavicans	BOLD:AAC0260	IBLAO1668-20	Hap_11	Spain	Huesca
Setina flavicans	BOLD:AAC0260	IBLAO1670-20	Hap_11	Spain	Gerona
Setina flavicans	BOLD:AAC0260	IBLAO1315-20	Hap_12	Spain	Huesca
Setina flavicans	BOLD:AAC0260	IBLAO1403-20	Hap_12	Spain	Huesca
Setina flavicans	BOLD:AAC0260	IBLAO1583-20	Hap_12	Spain	Lérida
Setina flavicans	BOLD:AAC0260	IBLAO534-12	Hap_12	Spain	Lérida
Setina flavicans	BOLD:AAC0260	IBLAO535-12	Hap_12	Spain	Lérida
Setina irrorella	BOLD:AAC0260	CGUKD808-09	Hap_12	United Kingdom	England
Setina irrorella	BOLD:AAC0260	ODOPE707-11	Hap_12	Germany	Oberbayern
Setina irrorella	BOLD:AAC0260	PHLAA036-09	Hap_12	Austria	Nordtirol
Setina irrorella	BOLD:AAC0260	LEATB559-13	Hap_13	Italy	Suedtirol
Setina irrorella	BOLD:AAC0260	LENOA1179-11	Hap_14	France	Haute Normandie
Setina irrorella	BOLD:AAC0260	LENOA1180-11	Hap_14	France	Haute Normandie
Setina irrorella	BOLD:AAC0260	GWORL290-09	Hap_15	Germany	Oberbayern
Setina aurita	BOLD:AAC0260	GWOTD800-12	Hap_16	Germany	South Bavaria
Setina aurita	BOLD:AAC0260	PHLAA003-09	Hap_16	Austria	Nordtirol
Setina aurita	BOLD:AAC0260	PHLAA009-09	Hap_16	Austria	Nordtirol
Setina aurita	BOLD:AAC0260	PHLAA033-09	Hap_16	Austria	Nordtirol
Setina irrorella	BOLD:AAC0260	LON3788-16	Hap_16	Norway	Aseral
Setina irrorella	BOLD:AAC0260	LON5531-17	Hap_16	Norway	Vang
Setina irrorella	BOLD:AAC0260	LON5534-17	Hap_16	Norway	Kristiansand
Setina irrorella	BOLD:AAC0260	PHLAA006-09	Hap_16	Austria	Nordtirol
Setina irrorella	BOLD:AAC0260	NOENO364-17	Hap_17	Austria	Niederoesterreich
Setina aurita	BOLD:AAC0260	PHLAA005-09	Hap_18	Austria	Nordtirol
Setina flavicans	BOLD:AAC0260	IBLAO1584-20	Hap_19	Spain	Lérida
Setina flavicans	BOLD:AAC0260	IBLAO1967-21	Hap_20	Spain	León
Setina flavicans	BOLD:AAC0260	IBLAO1957-21	Hap_21	Spain	Cantabria
Setina flavicans	BOLD:AAC0260	IBLAO1946-21	Hap_22	Spain	Cantabria
Setina flavicans	BOLD:AAC0260	IBLAO1665-20	Hap_23	Spain	Lérida
Setina flavicans	BOLD:AAC0260	IBLAO1314-20	Hap_24	Spain	Asturias
Setina flavicans	BOLD:AAC0260	IBLAO1313-20	Hap_25	Spain	Asturias
Setina irrorella	BOLD:AAC0260	PHLSA603-11	Hap_26	Italy	Chieti
Setina irrorella	BOLD:AAC0260	PHLAB1148-10	Hap_27	Italy	L'Aquila
Setina flavicans	BOLD:AAC0260	IBLAO1958-21	Hap_28	Spain	Cantabria

Taxon	BIN	Process ID	Haplotype	Country	Region
Setina flavicans	BOLD:AAC0260	IBLAO1311-20	Hap_29	Spain	Asturias
Setina flavicans	BOLD:AAC0260	IBLAO1310-20	Hap_30	Spain	Asturias
Setina irrorella	BOLD:ADE0333	LEALT291-16	Hap_31	Russia	Altai
Setina irrorella	BOLD:ADE0333	LEALT292-16	Hap_31	Russia	Altai
Setina irrorella	BOLD:ADE0333	LEALT293-16	Hap_31	Russia	Altai
Setina irrorella	BOLD:ADE0333	LEALT389-16	Hap_31	Russia	Altai
Setina irrorella	BOLD:ADE0333	LEALT391-16	Hap_31	Russia	Altai
Setina aurita	BOLD:ACF4655	PHLAA017-09	Hap_32	Switzerland	Val Bregaglia
Setina aurita	BOLD:ACF4655	PHLAI276-13	Hap_32	Austria	Vorarlberg
Setina irrorella	BOLD:ACF4655	PHLAA266-09	Hap_32	Austria	Vorarlberg
Setina irrorella	BOLD:ACF4655	PHLSA664-11	Hap_32	Austria	Vorarlberg
Setina irrorella	BOLD:ABZ5368	PHLAH800-12	Hap_33	France	Alps Marítims
Setina irrorella	BOLD:ABZ5368	PHLAH801-12	Hap_33	France	Alps Marítims
Setina aurita	BOLD:ABZ5368	PHLAH563-12	Hap_34	Italy	Cuneo
Setina aurita	BOLD:ABZ5368	PHLAH562-12	Hap_35	Italy	Cuneo
Setina aurita	BOLD:ABZ5368	PHLAA027-09	Hap_36	Italy	Brescia
Setina aurita	BOLD:ABZ5368	PHLAA028-09	Hap_36	Italy	Brescia
Setina aurita	BOLD:ABZ5368	PHLAA026-09	Hap_37	Switzerland	Val Poschiavo
Setina aurita	BOLD:ABZ5368	PHLAA013-09	Hap_38	Austria	Nordtirol
Setina aurita	BOLD:ABZ5368	LEATC118-13	Hap_39	Italy	Suedtirol
Setina aurita	BOLD:ABZ5368	LEATC119-13	Hap_39	Italy	Suedtirol
Setina aurita	BOLD:ABZ5368	LEATJ799-15	Hap_39	Austria	Nordtirol
Setina aurita	BOLD:ABZ5368	PHLAA010-09	Hap_39	Austria	Nordtirol
Setina aurita	BOLD:ABZ5368	PHLAA011-09	Hap_39	Austria	Nordtirol
Setina irrorella	BOLD:ACF5607	ABOLD152-16	Hap_40	Austria	Kaernten
Setina irrorella	BOLD:ABY4807	PHLAF321-11	Hap_41	North Macedonia	Mavrovo NP
Setina aurita	BOLD:ABZ4610	PHLAH564-12	Hap_42	France	Provence
Setina irrorella	BOLD:ABZ4610	PHLAH802-12	Hap_42	France	Provence
Setina aurita	BOLD:ABZ4610	PHLAA413-09	Hap_43	France	Provence
Setina alpestris	BOLD:ABZ4609	PHLAA047-09	Hap_44	Italy	Suedtirol
Setina irrorella	BOLD:ABZ4609	PHLAA293-09	Hap_44	Italy	Belluno
Setina irrorella	BOLD:ABZ4609	PHLAA294-09	Hap_44	Italy	Belluno
Setina irrorella	BOLD:ABZ4612	LEFID143-10	Hap_45	Finland	Ostrobottnia
Setina irrorella	BOLD:ABZ4612	LEFIF133-10	Hap_45	Finland	Ostrobottnia

To clarify the taxonomic status of several *Setina* taxa, two molecular species delimitation methods were applied to identify potential Molecular Operational Taxonomic Units (MOTUs) that may correspond to putative cryptic species. Both approaches were distance-based. The first was the Barcode Index Number (BIN) System (Ratnasingham and Hebert 2013), and a hierarchical clustering algorithm Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021). The BIN method (Ratnasingham and Hebert 2013) is implemented within the BOLD. Newly submitted sequences are automatically compared with those already available in BOLD and clustered according to their molecular divergence. This clustering is preformed using algorithms designed to detect discontinuities among sequence groups, with each cluster assigned a unique and globally recognized BIN identifier within BOLD. The BIN pipeline analyzes barcode-region sequences as soon as they are uploaded to the database. BIN metadata are dynamic, as key elements of specimen records—particularly taxonomic identifications—are frequently updated by data contributors, and the continuous addition of new records may lead to revisions of existing clusters (Ratnasingham and Hebert 2013). The ASAP method (Puillandre et al. 2021) infers species partitions from single-locus sequence alignments, such as DNA barcode datasets. Although conceptually grounded in evolutionary theory, ASAP is implemented as a hierarchical clustering algorithm that relies solely on pairwise genetic disparities. This approach avoids the computational demands of phylogenetic reconstruction and process species partitions without relying on prior biological knowledge of intraspecific variation. According to Puillandre et al. (2021), ASAP has demonstrated greater performance than several previously used species delimitation methods, including the barcode-gap approach Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012), the General Mixed Yule-Coalescent model (GMYC) (Pons et al. 2006), and the Poisson Tree Process (PTP) (Zhang et al. 2013). The first two methods were originally developed within a maximum likelihood framework, whereas later developments extended these approaches into a Bayesian framework (Reid and Carstens 2012).

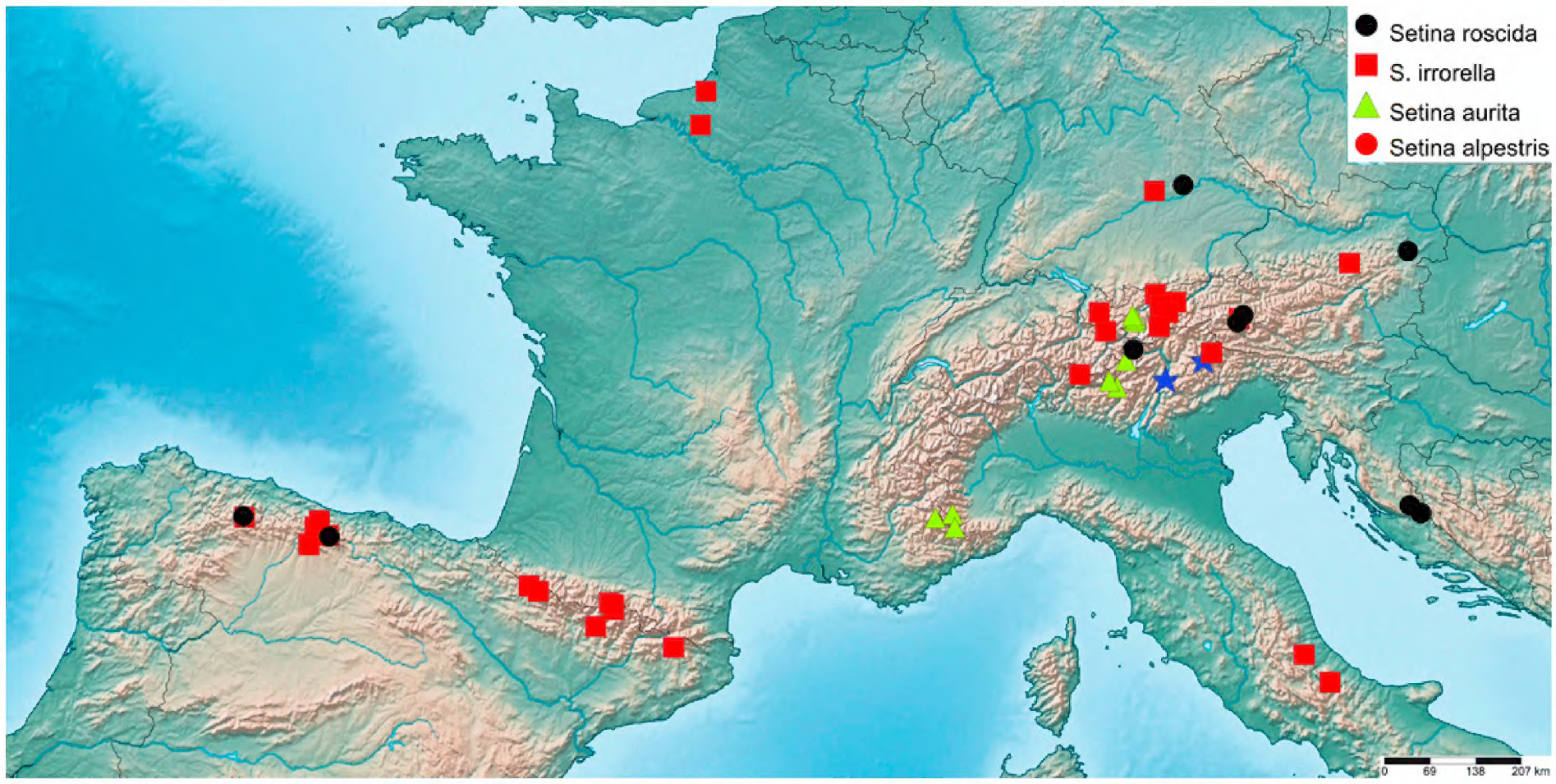

Figure 1. Distribution of *Setina* samples sequenced in Western-central Europe. Note that each point may represent more than one individual. The map was created using www.simplemappr.net.

Results

Molecular analysis and BIN congruence

In the dataset composed of 87 sequences, 86 individuals of *Setina* were sequenced, or their conspecific sequences were acquired from the database (BOLD) to analyse their taxonomic identification with geographical species grouping.

The mean K2P values between the morphologically determined species were used to study species delimitation using two different methods: BIN and ASAP. The BIN System is an online framework that clusters barcode sequences algorithmically and is recalculated from time to time as the number of sequences of each species increases. All the COI sequences from the *Setina* lineages were uploaded and examined into the Barcode of Life Data System (BOLD), with a result of ten BINs (BOLD:AAC0260, BOLD:ABZ1427, BOLD:ABZ5368, BOLD:ACF4655, BOLD:ABZ4610, BOLD:ADE0333, BOLD:ACF5607, BOLD:ABY4807, BOLD:ABZ4612, BOLD:ABZ4609). In total, 45 haplotypes were found in the 86 barcode sequences analysed of the ten BIN lineages of *Setina* taxa, ranging from twenty-one haplotypes in BIN BOLD:AAC0260 to an unique haplotype in BINs BOLD:ADE0333, BOLD:ACF5607, BOLD:ABY4807 and BOLD:ABZ4612, for individuals identified as *S. irrorella*. The largest BIN BOLD:AAC0260 includes individuals initially identified as *S. irrorella*, *S. aurtia* and *S. flavicans*, and other as BIN BOLD:ABZ1427 includes nine haplotypes for *S. roscida* and *S. cantabrica* or seven haplotypes in BIN BOLD:ABZ5368 including individuals of *S. irrorella* and *S. aurita* (Table 1). The overall haplotype diversity (Hd) was 0.9718 ± 0.007 in 78 polymorphic sites with 78 mutations and nucleotide diversity per site (Pi) was 0.01771. No insertions or deletions were found.

A phylogenetic hypothesis with Maximum Likelihood (ML) generated using FastTree 2.1.11 software was chosen as the basis for our discussion (Fig. 2) and the monophyly of *Setina* species was recovered with the inclusion of *Stigmatophora micans* as outgroup to root the tree by ML (Fig. 2) and NJ methods (Fig. 3).

Three clusters were found and were thereafter treated as three MOTUs or putative species, namely *S. roscida* (BOLD:ABZ1427), *S. irrorella* (BOLD:AAC0260) and *S. aurita* (BOLD:ABZ5368, BOLD:ACF5607, BOLD:ACF4655, BOLD:ADE0333, BOLD:ACF4609, BOLD:ABZ4610). Only one cluster (BOLD:ABY4807, BOLD:ABZ4612), including species initially misidentified *S. irrorella* was in a group separated from the rest in ML and in a *S. aurita* group in NJ trees. Samples initially identified as *S. alpestris* with BIN ACF4609 and *S. irrorella* from Russia with BIN ADE0333 were clustered together with the main *S. aurita* group. Divergence between the four accepted species including *S. alpestris* varies between 1.7% and 2.9% (mean 2.1%; Table 2). The highest interspecific value was found between *S. roscida* and *S. alpestris* (2.9%) and between *S. irrorella*-*S. roscida* and *S. irrorella*-*S. alpestris* (2.5% and 2.3%, respectively), whereas the lowest ones were found between *S. aurita* and all the others (mean 1.7%). The total number of nucleotide substitutions between species is 78 variable sites in the overall sample (Table 2, Suppl. material 1).

In order to examine the taxonomic status of *S. cantabrica* and *S. flavicans* accepted by some authors, four samples labeled IBLAO822-12, IBLAO1316-20, IBLAO1317-20, IBLAO1949-21 and IBLAO1959-21 from the Cantabrian Mountains (table 1) have been found to fully correspond by morphological study and distribution pattern with *S. cantabrica*. Similarly, eighteen samples labeled IBLAO534-12, IBLAO535-12, IBLAO1310-20, IBLAO1311-20, IBLAO1313-20, IBLAO1314-20,

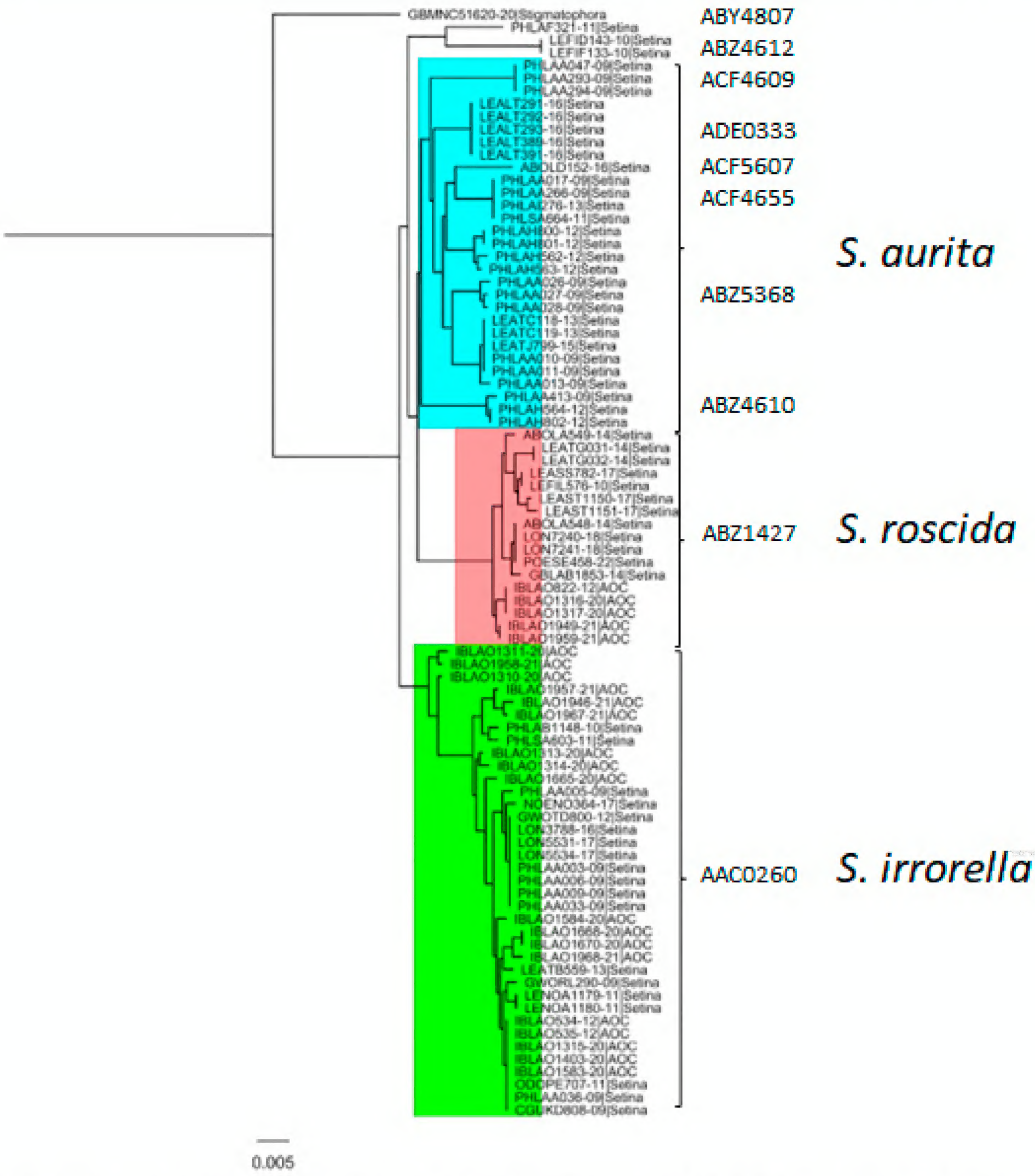

Figure 2. Maximum Likelihood tree obtained from 86 nucleotide COI sequences. The depth of each branch shows divergence within lineages. The scale bars represent 0.005 genetic difference.

IBLAO1315-20, IBLAO1403-20, IBLAO1583-20, IBLAO1584-20, IBLAO1665-20, IBLAO1668-20, IBLAO1670-20, IBLAO1946-21, IBLAO1957-21, IBLAO1958-21, IBLAO19678-21, and IBLAO1968-21 from the Cantabrian Mountains to the Pyrenees (table 1) were also identified as *S. flavicans*. Their sequences were compared in the reference library of lepidopteran barcodes using the identification engine (BOLD-ID), and we found that they matched sequences of *S. roscida* and *S. irrorella* from Central Europe, respectively. The values obtained between *S. irrorella* and *S. flavicans* varied from 0 (full match) to 0.15% and between *S. roscida* and *S. cantabrica* was 0.38% (Table 2). These values are lower than the average among *Setina* species studied (2.1%).

The *Setina* cluster showing three main different subgroups with genetic differences between 1.7% and 2.9% belong to *S. roscida*, *S. irrorella* and *S. aurita* (Figs 2, 3). The *S. roscida* cluster (n = 17; BIN BOLD:ABZ1427) included individuals identified as *S. roscida* and the subspecies *S. r. cantabrica* and *S. r. kuhlweini* Hübner, 1824

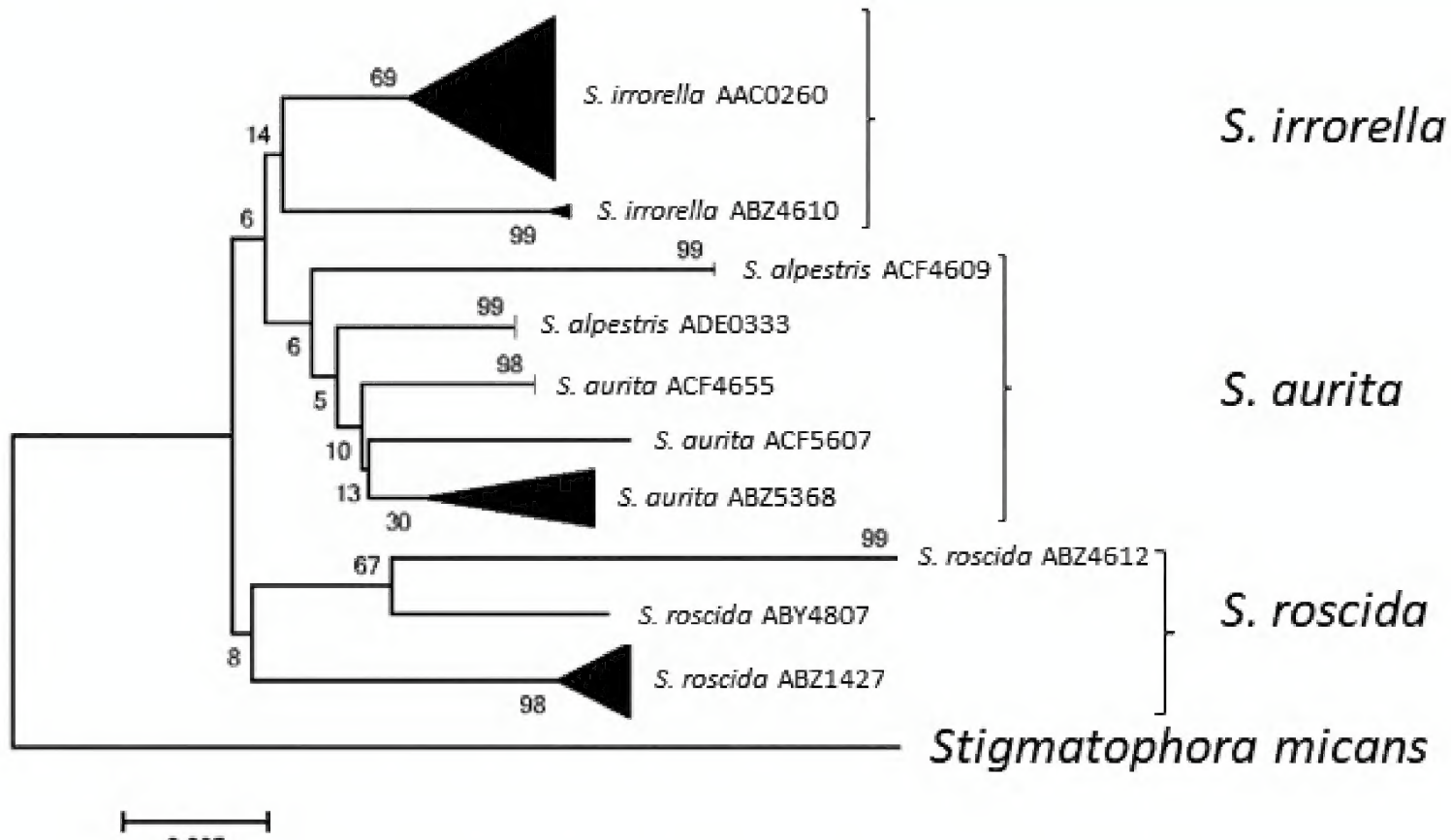

Figure 3. Neighbour-Joining tree obtained from 87 nucleotide COI sequences. The depth of each branch shows divergence within lineages. Bootstrap values are provided at major nodes. The scale bar represents 0.01 genetic difference.

Table 2. Interspecific mean K2P (Kimura 2-Parameter) divergences (mean pairwise distances) based on the analysis of COI fragments (>500 bp) among *Setina* species including subspecies *flavicans* and *cantabrica* previously considered as species.

	S. alpestris	***S. aurita***	***S. flavicans***	***S. irrorella***	***S. cantabrica***	***S. roscida***
S. alpestris		1.75	2.40	2.30	2.8	2.96
S. aurita			1.59	1.75	1.75	1.80
S. flavicans				0.15	2.28	2.36
S. irrorella					2.40	2.50
S. cantabrica						0.38
S. roscida						

(Table 2, Suppl. material 1; Process ID POESE458-22) with similar mean genetic differences (0.3-0.4%) and with maximum values between *S. roscida-S. r. cantabrica* (0.8%), *S. roscida-S. r. kuhlweini* (0.6%) and *S. r. cantabrica-S. r. kuhlweini* (0.5%) (Table 2, Suppl. material 1).

The *S. aurita* cluster (n = 22; BINs BOLD:ACF4655, BOLD:ABZ5368, BOLD:ADE0333, BOLD:ACF5607, BOLD:ABZ4609) included five individuals belonging to BIN BOLD:ADE0333 initially identified as *S. irrorella* from Russia, four individuals belonging to BIN BOLD:ACF4655 identified as *S. aurita* and *S. irrorella* from Austria and Switzerland, one individual in BIN BOLD:ACF5607 probably misidentified as *S. irrorella* with 0.9% mean genetic difference with BOLD:ABZ5368, and the BIN BOLD:ABZ4609 (n = 3) with specimens identified as *S. alpestris* and *S. irrorella*.

The *S. irrorella* cluster (n = 37; BIN: BOLD:AAC0260) included individuals initially identified as *S. irrorella*, *S. i. flavicans* and *S. aurita* with 0.15% mean genetic difference between *S. irrorella* and *S. i. flavicans*, and the subgroups BIN BOLD:ABY4807 (n = 1) from North Macedonia, and BIN BOLD:ABZ4612 (n = 2) from Finland.

Other groups differentiated from the three main clusters were BIN BOLD:ABZ4610 (n = 2) indistinctly identified as *S. aurita* and *S. irrorella* from Provence (France).

Delimitation of species

The ASAP method was performed on the data set of 87 sequences, representing all individuals sequenced including *Stigmatophora micans* as outgroup. The method calculates the probability value (p-value) that each of the new groups represents a single species, and the rank calculates the width of the barcode gap between these groups. Both metrics are combined into a single ASAP-score that is used to rank the partitions. The graphical output shows each node of the hierarchical grouping with the same probability of being a panmictic species ($p > 0.1$) (Table 3, Fig. 4). In our analysis, ASAP-score 2.0 was the smallest within the range of genetic distances calculated as the average among the third largest p-value (0.739) and the smallest rank of relative barcode gap width (1.91e-04). Two MOTUs for groups of species were identified: *Setina* and *Stigmatophora*. The next smallest ASAP-score was 3.0 calculated as the average among the second largest p-value (0.110) and the fourth smallest rank of relative barcode gap width (3.90e-04). This analysis resulted in partitioning all COI sequences into thirteen MOTUs (hypothetical species) corresponding to the *Stigmatophora micans* and the three main *Setina* lineages: *S. roscida*, *S. aurita* (5 groups), and *S. irrorella* (5 groups).

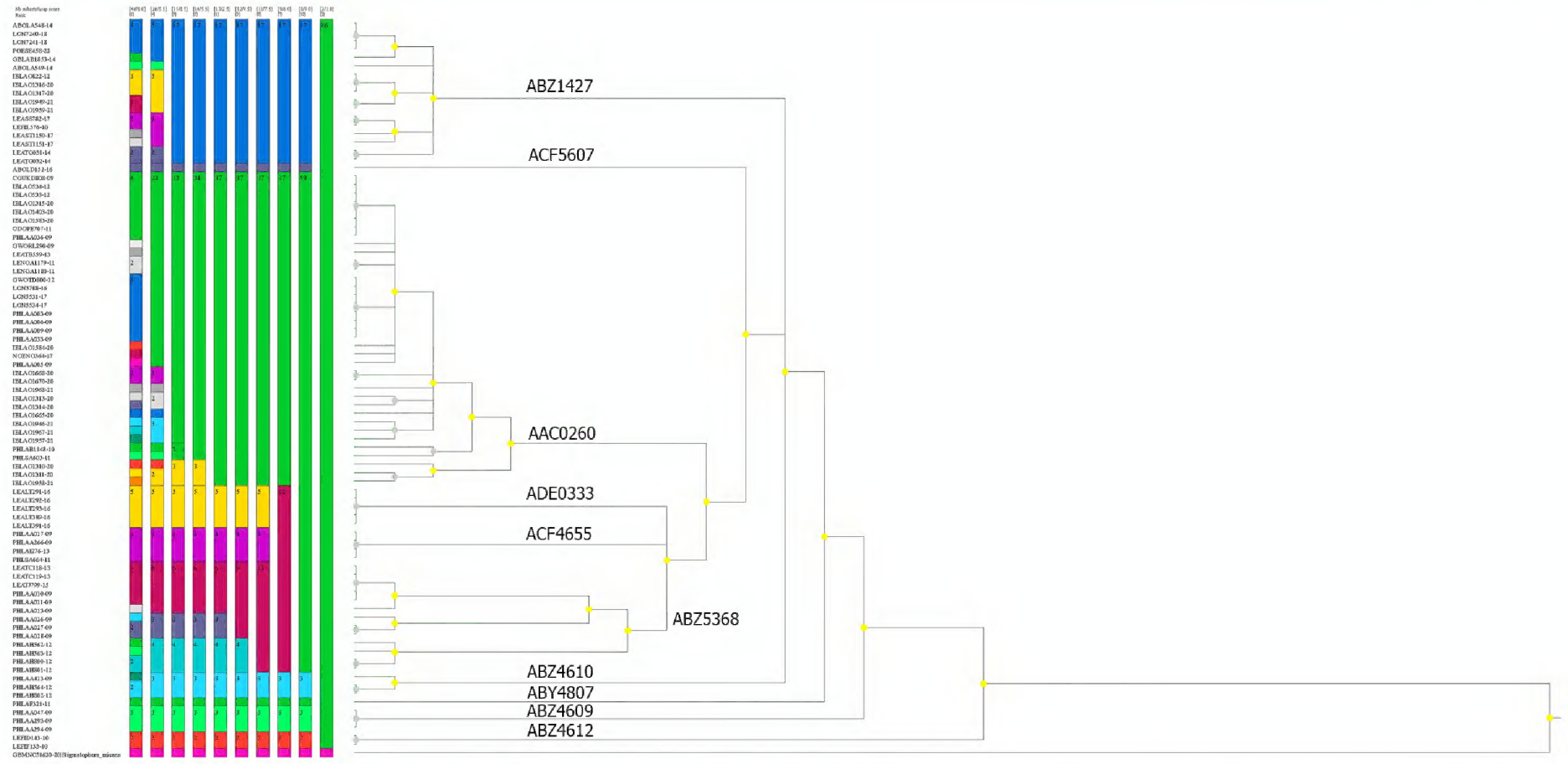

Figure 4. ASAP tree of *Setina* species, obtained from 87 nucleotide COI sequences, with *Stigmatophora micans* as outgroup to root the tree. The depth of each branch shows divergence within lineages. BINs for groups are indicated for each node. ASAP species delimitation results are indicated by vertical and coloured bar.

Additionally, the ASAP method also differentiated the 46 haplotypes previously calculated with the ASAP-score value of 7.0 (average of 0.867 p-val and 1.20e-05 relative barcode gap width) (Table 3).

The two methods used for species delimitation allow recognition of the three main lineages as distinct from any other groups of *Setina* species studied. The exceptions are the sequences found outside of major clusters identified as *S. aurita* with BINs BOLD:ABZ4610 collected in France and those identified as *S. irrorella* with BIN BOLD:ABY4807 from North Macedonia, BOLD:ACF5607 from southern Austria and BOLD:ABZ4612 from Finland. These groups show more than 3.8% of genetic differentiation with the main *Setina* groups and should be studied separately. A BOLD:ABZ4609 group was also separated including individuals indistinctly identified as *S. alpestris* and *S. irrorella* but without special grouping as other BINs (Fig. 4).

Table 3. Species partitions from COI sequence alignments based on ASAP method with number of species or MOTUs delimited (nr species), ASAP-score calculated between P-val (probability of panmixia) and W (relative gap width metrics) with their respective ranks given in parenthesis and, d_t as threshold distance defined as the mid-point within the current distance. The lower the ASAP-score, the better the partition.

Nr species/ MOTUs	ASAP-score	P-val (rank)	W (rank)	d_t
* 2	2.00	7.39e-01 (3)	1.91e-04 (1)	0.036034
* 13	3.00	1.10e-01 (2)	3.90e-05 (4)	0.007631
12	4.00	8.64e-01 (6)	5.64e-05 (2)	0.009932
9	6.50	8.70e-01 (7)	2.74e-05 (6)	0.013016
46	7.00	8.67e-03 (1)	1.20e-05 (13)	0.000760
26	7.00	7.68e-01 (4)	1.52e-05 (10)	0.002283
14	7.00	7.78e-01 (5)	1.60e-05 (9)	0.005335
27	7.50	8.74e-01 (8)	2.02e-05 (7)	0.001522
11	8.00	9.70e-01 (11)	3.73e-05 (5)	0.011469
8	8.50	9.82e-01 (14)	5.58e-05 (3)	0.014566

Discussion

The genus *Setina* is a species-group with a great inter-individual and inter-population variability, whose species often show a similar type and range of intra- and interspecific variation in external features, and with the male genitalia very uniform throughout the genus. Furthermore, the alleged morphological specific features

are usually only slightly different. Only According to Witt and Ronkay (2011), differential features are found in the female genitalia, where the shape and size of the specialized ductus bursae and the appendix bursae define the species-rank taxa. Thus, relatively high degree of hybridization in sympatric species cannot be ruled out. In fact, hybridization may partly explain the variation in external features observed (Burmann, 1955; Burmann & Tarmann, 1985).

Leraut (2018) analyzed extensive samples of European populations of *Setina* species and concluded that, according to the study of their biology, as well as that of the external characters and the genitalia, the genus *Setina* in Europe was separated into four species, named *S. roscida*, *S. aurita*, *S. irrorella* and *S. alpestris*, which tend to vary by constituting more or less locally homogeneous populations, with crosses between species that could lead to hybridization and introgression in the contact zones.

Detailed morphological, ecological and genetic analyses may discriminate closely related species which possess slight sequence divergence from their nearest neighbour. Molecular analyses enable initial taxonomic evaluation in such taxa, but there is no objective way to select the algorithm for species delimitation or input parameters that best recover actual species boundaries when using barcodes only. In different groups of invertebrate taxa, a sequence divergence in the barcode region lower than 2% is assumed by many authors to correspond to intraspecific differences, while higher values of divergence are related to interspecific variation and groups of sequences are assumed to correspond to distinct MOTUs (Hausmann et al. 2011; Zhang and Bu 2022). However, divergence between young sister-species may fall below the 2% threshold, while unusually variable species may exceed it (Hausmann et al. 2011; Zhang and Bu 2022).

Discrimination of differences affecting these species requires more algorithmic finesse, and the selection of an effective algorithm for MOTU recognition is necessary. In our study BIN and ASAP methods were selected to analyze the *Setina* sequences. Barcode Index Number (BIN) system is a consistent and reasonably stable registry for animal MOTUs, recognized through sequence variation in the barcode region. Concerning the species delimitation analyses by ASAP, Puillandre et al. (2021) assessed its power on real COI barcode data sets, noticing the ASAP potential to become a relevant tool to test hypothesis on maternal ancestry in a barcode-based integrative taxonomy process. ASAP identifies a taxonomic partition ranked with different scores that must be subsequently tested against other evidence in an integrative taxonomy framework, especially when single-locus data are used.

In our study, BOLD calculated ten different BINs for all taxa, of which *S. roscida* has a single BIN, while *S. aurita* and *S. irrorella* have multiple BINs, perhaps as a result of hybridization. Similarly, the ASAP method for the barcoding data obtained in our study indicated the existence of the same three main clusters of *Setina* distributed from the Iberian Peninsula to Russia. The variation in the BIN assignments and the several branches calculated by ASAP for *S. irrorella* and *S. aurita* within or without the main groups suggest the inability of sequence variation at COI to diagnose other species.

This variability was noticed initially by Toulgoët (1975), remarking the general conformation of the genitalia, wings, venation, pattern, tymbal organs and coloring as basically identical in all representatives of the genus, and caterpillars show no differences in general aspect and coloration. In relation to this variation, Trawöger (1991) explained the polymorphism of *S. aurita* and *S. irrorella* populations by the reciprocal gene flow between the two species, which produced specimens with typical traits of the other species. This variability was also recorded by Witt and Ronkay (2011), listing several subspecies in each of their recognized species: four subspecies for *S. aurita*, -*S. a. aurita*, *S. a. imbuta* (Hübner, 1803), *S. a. pfisteri* Burmann & Tarmann, 1985 and *S. a. teriolensis* (Burmann, 1955)-, three subspecies for *Setina irrorella* -*S. i. irrorella*, *S. i. freyeri* (Nickerl, 1845), *S. i. mediterranea* (Daniel, 1964), two subspecies for *Setina flavicans* -*S. f. flavicans* and *S. f. pseudoirrorella* de Freina & Witt, 1985-, two subspecies for *Setina roscida* -*S. r. roscida* and *S. r. kuhlweini* (Hübner, 1824)- and only *Setina cantabrica* was separated as a different species in the *roscida* species group. Additionally, these authors considered three subspecies of *Setina alpestris*: *S. a. alpestris* Zeller, 1865, *S. a. wolfsbergeri* (Burmann, 1975) and *S. a. pseudokuhlweini* (Vorbrodt, 1914).

The taxonomic status of all these nominal taxa has been discussed by different lepidopterists. *S. roscida cantabrica* and *S. irrorella flavicans* were previously examined by de Freina and Witt (1985) based on *Setina* individuals from Spain and southern France and they concluded that *S. irrorella* was not present in the Iberian Peninsula, being replaced by *S. flavicans*. In their opinion, individuals from the French Pyrenees and northern Spain could be distinguished as a new subspecies, *S. flavicans pseudoirrorella*. Witt and Ronkay (2011) pointed out the differences between the male genitalia of the species: *S. flavicans* can be distinguished from those of *S. alpestris* by the dorsally more flattened uncus, shorter and apically more curved digitus, the longer clasper and the more robust basal cornutus of the vesica; *S. roscida*-*S. cantabrica* pair of species by the dorsally less arched uncus, much broader valvae with longer and more acute digitus and the shorter, thicker clasper, and *S. irrorella* and *S. ramosa* by some slighter and more variable characteristics when large series of individuals are studied as the more robust, medially evenly thick and distally less curved uncus, the longer and apically more curved digitus, the longer, thicker clasper and the larger basal cornutus of the vesica. The female genitalia of *S. flavicans* are most similar to those of *S. irrorella* while the differences with *S. alpestris*, *S. aurita* and *S. roscida*

are conspicuous. *Setina flavicans* is considered by de Freina and Witt (1987), Ylla et al. (2010) and Witt and Ronkay (2011) as a distinct species while Leraut (2006) and Leraut (2018) considered nominotypical *S. flavicans* to be conspecific with *S. irrorella*.

In relation to *Setina cantabrica* de Freina and Witt (1985) described it from the Picos de Europa in Asturias and, later on, Fernández-Vidal et al. (2003) extended its known range to north-west Spain, finding it in León, Lugo and Orense as well as in Asturias, where the species flew sympatrically in some places with *S. flavicans*. Corley (2013) studied some individuals from Portugal previously identified as *S. flavicans*, pointing out that their genitalia best match with those of *S. roscida*, leading to the conclusion that the placement of Leraut (2006) of *S. cantabrica* as a subspecies of *S. roscida* may well be correct although the Portuguese specimens (28 mm) are all larger than typicall *S. roscida* (20-24 mm). Subsequently, Leraut (2006) downgraded *S. cantabrica* to a subspecies of *S. roscida* based on the lack of significant differences in the male genitalia, although Witt and Ronkay (2011) considered that these differences are distinctive within the genus *Setina*. Leraut (2018) considered *S. cantabrica* as a subspecies of *S. roscida* considering the comments on non-significant differences in genitalia and within the variation between different subspecies of Leraut (2006) and Corley (2013).

The current distributions of *Setina* species are the result of multiple range shifts driven by past climatic changes, that profoundly influenced the biodiversity of Europe (for reviews, see e.g., Hewitt 1996, 1999, 2000, 2004; Taberlet et al. 1998; Schmitt 2007). Initially, *S. roscida* was considered a steppe species and *S. aurita* a rock dweller while *S. irrorella*, thought to be the probable ancestor of all, is the least specialized and the most widespread, inhabiting almost any biotope from sea level to 3,500 m above sea level. The *Setina* species, which allegedly migrated from the Eastern European steppes to the mountainous regions of Europe before the last Ice Age, were split up into numerous, mostly genetically isolated populations in the Alps, Pyrenees and Balkans. The ecologically uniform steppes of the east contrast with the conditions found at different altitudes in the valleys, mainly in the alpine region, which has led to the development of slight morphological divergence between populations. This has led to the description of numerous infraspecific taxa by authors because the mechanisms of genetic, ethological and ecological isolation have been not fully developed, allowing hybridization in areas of contact (Toulgoët 1975; de Freina and Witt 1984, 1985; Trawöger 1991, 1994; Parenzan and Scalercio 2001; Leraut 2018).

Distributional patterns are likely related to the isolation of populations in refugia that promoted genetic and phenotypic differentiation as a result of independent adaptation to local environments and genetic drift, with consequences for reproductive isolation between discrete refugial lineages and the creation of hybrid zones where diverged lineages came into secondary contact, especially in the Alpine regions (Capblancq et al. 2020; Augustijnen et al. 2022). The major lineages of *Setina* are represented by two widespread groups distributed throughout Europe namely *S. irrorella* from Spain to European parts of Russia and *S. roscida* from Iberian Peninsula to western and central Siberia, and *S. aurita* restricted to the entire Alps. Regarding *Setina alpestris*, generally characterized by the absence of the black suffusion on the underside of the forewings, it is often misidentified as *S. ramosa*, and its taxonomic status and distribution remains to be clarified, as also suggested by Leraut (2006). Trawöger (1994), comparing the variation between *S. alpestris* and *S. aurita*, suggested that the taxonomic status of *S. alpestris* cannot be recognized as a species despite the morphological and ethological differences and it could be considered as "semispecies" or species in "statu nascendi".

All the species show a morphological pattern that could be considered evidence for similar ecological preferences or parallel histories during the Quaternary, suggesting that all groups share a recent common ancestor. This phylogeographic pattern has been suggested for other Lepidoptera (e.g., *Agrodiaetus*, *Erebia*, *Melitaea*, *Parnassius*, *Chelis*, *Arctia*), in which a marked role of climatic oscillations during the Pleistocene on population isolation and differentiation is postulated (Haubrich and Schmitt 2007; Todisco et al. 2012; Albre et al. 2008; Lenevea et al. 2009; Vila et al. 2010; Ortiz et al. 2016, 2023).

However, an increasing number of studies indicate that many endemic taxa inhabiting refugial regions are of Pleistocene origin and formed by allopatric fragmentation (e.g., Hewitt 1996; Ghanavi et al. 2025; Ortiz et al. 2016, 2023; Torrado-Blanco et al. 2024). The apparent absence of a clear lineage sorting between *Setina* species may be due relatively recent radiation. In some cases, they are described as distinct species and, in other cases, these taxa are considered as subspecies or lineages within species. The present occurrence of multiple morphs of species showing a sympatric and parapatric distribution is thought to be the result of a range expansion of *S. irrorella* and *S. roscida* since the last glaciation events, thanks to the high ecological plasticity of these species and the finding of many suitable environments. In addition, the presence of individuals with different phenotypes within the groups of particular species suggest hybridization processes between the species that cause a great morphological variability that can lead to misidentification.

We admit that the approach based on the barcode patterns of *Setina* species is of limited power for the resolution of their taxonomy as biospecies, but it is a contribution towards the understanding of the genus grouping the studied taxa into three species, *Setina irrorella* (Linnaeus, 1758), *Setina roscida* (Denis & Schiffermüller, 1775) and *Setina aurita* (Esper, 1787). However, this grouping should not be regarded as a phylogeny, before getting confirmation from a comprehensive molecular

phylogenetic study. This morphological grouping should rather serve as a tool for species identification, regardless of the subspecies considered within each species or any new taxa that may be described.

As a final tale, we shall note that the specific name *Setina aurita*, as used here, should be reviewed, given the aforementioned homonymies.

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Supplementary material 1

Interspecific K2-P distances 86 COI sequences

Authors: Antonio S. Ortiz
Data type: xlsx
Explanation note: Similarity index matrix by COI K2-P distances for 86 sequences of Setina species studied. The metadata of each sample ID can be accessed in dx.doi.org/10.5883/DS-SETINA in BOLDSYSTEM.
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